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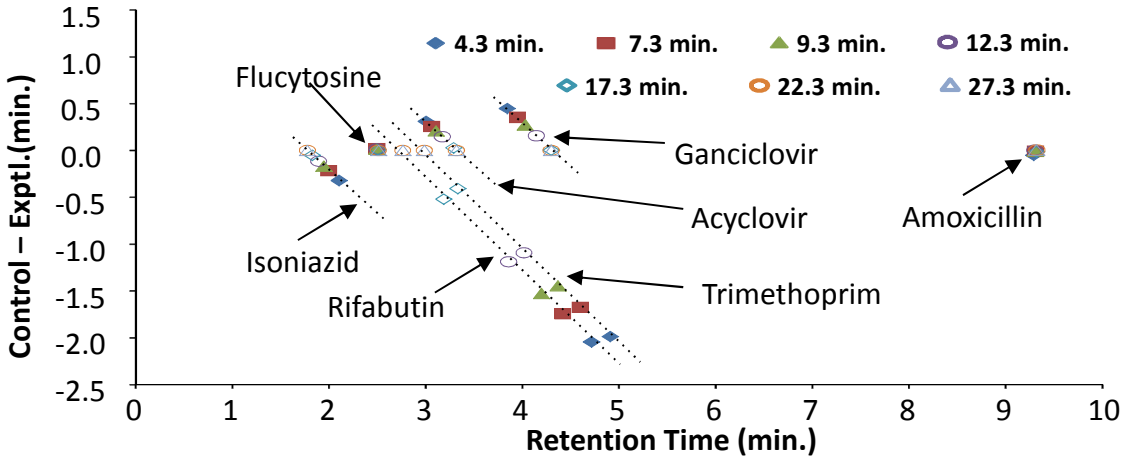
4.3 min.
7.3 min.
9.3 min.
12.3 min.
17.3 min.
22.3 min.
27.3 min.
Flucytosine
Ganciclovir
Acyclovir
Amoxicillin
Isoniazid
Rifabutin
Trimethoprim
Control – Exptl.(min.)
Retention Time (min.)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
1
2
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Retention characteristics of some antibiotic and anti-retroviral compounds in hydrophilic interaction chromatography using isocratic elution, and gradient elution with repeatable partial equilibration.

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Abstract

The separation of some zwitterionic, basic and neutral antibiotic and antiretroviral compounds was studied using hydrophilic interaction chromatography (HILIC) on bare silica, bonded amide and urea superficially porous phases. The differences in the selectivity and retentivity of these stationary phases were evaluated for compounds with widely different physicochemical properties (logD -3.43 to 2.41 at $_{w}^{w}$pH 3.0). The mobile phase was acetonitrile-ammonium formate buffered at low $_{w}^{w}$pH. Compounds containing quinolone and serine groups showed poor peak shapes on all columns, attributed to metal-oxide interactions with system metals. Peak shapes were improved by addition of citrate buffers. Gradient elution, particularly with regard to column equilibration, was also studied due to the large differences in retention factors observed under isocratic conditions. Full equilibration in HILIC was slow for both ionogenic and neutral solutes, requiring as much as ~40 column volumes. However, highly repeatable partial equilibration, suitable for gradient elution, was achieved in only a few minutes. Pronounced selectivity differences in the separations were shown dependent on the partial equilibration time.

Keywords: HILIC; antibiotics; antiretrovirals; peak shape; gradient elution.

1. Introduction

Hydrophilic interaction chromatography (HILIC) is becoming widespread in application areas such as pharmaceutical [1], metabolite profiling [2,3], clinical [4] and environmental analysis [5]. It is a particularly useful technique when dealing with highly polar and/or ionogenic compounds that can give rise to poor retention or peak shape in reversed-phase chromatography [6]. In HILIC, retention is thought to be due to varying combinations of partitioning, electrostatic (ionic) and adsorption interactions [7]. Usually, the mobile phase is a water-miscible aprotic solvent such as acetonitrile (typically > 60% v/v) combined with a soluble aqueous buffer. It is now widely accepted that a major retention contribution is partitioning that occurs between a pseudo-immobilised water layer that persists at the stationary phase surface and the bulk mobile phase [8–11]. Ionic and adsorption interactions can also exist between free silanol and/or polar bonded groups on the stationary phase with charged moieties and hydrogen bonding sites on the solute. Attempts have been made to identify the differences between HILIC stationary phases in order to elucidate those that are of most use to the practitioner [12–14]. Furthermore, attempts at modelling retention [15,16] in HILIC have been made in order to facilitate optimisation and method development. HILIC has many advantages over RP such as improved desolvation and sensitivity with nebuliser-based detectors [17–20], lower operating pressures at a given linear velocity [6,21], superior peak shapes and column performance with basic compounds [6,22] as well as the possibility to achieve significantly different selectivity [23].

HILIC is a useful technique in the clinical laboratory, particularly with regards to therapeutic drug monitoring (TDM) [4,24]. TDM is necessary for obtaining accurate patient serum concentrations of a given drug in order to optimise the dosage levels; this ensures maximum efficacy as well as minimizing the potential for adverse toxic events. Adams *et al*. [25] reviewed the adoption of HILIC for the measurement of aminoglycoside, β-lactam and tetracycline antibiotics. They noted that these classes of compounds were very hydrophilic, suggesting that HILIC was highly appropriate for their analysis. Liquid chromatography combined with either mass spectrometry (LC-MS), fluorescence (FL) or ultraviolet detection (UV) is now widely adopted for TDM in many clinical laboratories. The main advantages of LC-MS for TDM are regarded as due to improved specificity and sensitivity compared with immunoassay [26]. In particular, antibiotic and antiretroviral drugs represent a class of medicines that need to be closely monitored for establishing efficacy in cases of multi-drug resistant infections and indeed also to monitor for patient compliance. One of the main analytical challenges with monitoring these compounds is that their physiochemical properties (i.e. logP, logD and pK_a) vary widely; therefore it is important to select the most appropriate technique for obtaining good

chromatographic performance, retention and selectivity. This is particularly relevant when the monitoring of a drug of interest must be separated from isobaric interference [27]. For example, certain antiretroviral compounds are closely related to highly polar, endogenous nucleoside and nucleobase compounds such as uridine and cytosine. Often, antiretroviral and antibiotic therapies are administered in combination, making the choice for suitable chromatographic conditions for their measurement by a single method difficult.

One of the main chromatographic difficulties when dealing with a sample containing compounds with widely different physicochemical properties is that gradient elution is usually required. Ultimately, the aim of any devised method is to provide adequate throughput in a reasonable time frame, while maintaining chromatographic resolution, at least at an adequate level for LC-MS. The adoption of gradient elution methods can be problematic, as the repeatability of the method can be compromised by the requirement to re-equilibrate the column between runs. This obviously limits the throughput of the procedure. In RP, around 20 column volumes of the initial eluent are required to reach full thermodynamic equilibrium [28]. However, excellent run-to-run repeatability has been demonstrated with only 2 column volumes of mobile phase whereas the time for full equilibrium can be reduced with the co-addition of ancillary solvents [28–30]. Gradient methods are often performed in HILIC, yet the amount of initial eluent required to achieve full equilibration is often quoted only anecdotally. However, it is believed that equilibration in HILIC takes around twice that of RP, yet very little data exists to substantiate these claims.

The aim of this study was to investigate the applicability of HILIC for a range of physicochemically different antibiotic and antiretroviral compounds. These included antibiotic compounds mainly used in the treatment of tuberculosis: rifamycins (rifabutin/rifampicin), oxazolidazone (linezolid), beta-lactams (amoxicillin, flucoxacillin, meropenem, penicillin G, piperacillin, tazobactam), fluoroquinolones (ciprofloxacin/moxifloxacin), pyrimidine analogue (flucytosine), chloramphenicol, isoniazid, pyrazinamide, d-cycloserine, trimethoprim and sulfamethoxazole. The antiretroviral compounds studied were the guanosine analogues (acyclovir/ganciclovir). We chose these compounds partially due to their wide clinical usage, range of log D values (mostly moderately positive to negative values, indicating potentially satisfactory retention in the HILIC mode) and also for the presence of UV chromophores. The work could be extended to other important classes of antibiotics such as aminogylcosides, however, due to their lack of appreciable conjugation would be more suited to mass spectrometric detection, which was not used in the present study. We initially determined which compounds were amenable to HILIC in terms of retention and selectivity by comparing bare silica, amide and urea phases. The bonded

phases were based on the same superficially porous particles as the bare silica phase. We also wished to investigate peak shape effects for compounds containing certain functional groups. Finally, we performed a detailed study of gradient re-equilibration in HILIC, comparing bonded (urea and amide functionalised) and un-bonded HILIC phases at both low and moderate buffer concentrations adjusted to $_{w}^{w}$pH 3. Such a study highlights an important practical aspect for adopting HILIC in routine laboratories. This work builds on previous findings of column equilibration in HILIC both in isocratic and gradient modes [31,32].

2. Materials and Methods

2.1 Chemicals and reagents

Acetonitrile (HPLC gradient grade), ammonium formate (AF), ammonium citrate tribasic (AC), formic acid and toluene were purchased from Fisher Scientific (Loughborough, UK). D-Cycloserine, Rifampicin, Chloramphenicol, Ciprofloxacin, Isoniazid, Sulfamethoxazole, Amoxicillin, 5-Fluorocytosine, Penicillin G sodium salt, Piperacillin sodium salt and Trimethoprim were obtained from Sigma-Aldrich (Poole, UK). Flucloxacillin sodium salt was from EDQM, European Pharmacopoeia (Strasbourg, FR). Tazobactam sodium salt was from MicroConstants, Inc. (San Diego, USA). Moxifloxacin HCl from Bayer Pharma AG (Wuppertal, DE). Pyrazinamide, Rifabutin, Meropenem, Ganciclovir, Linezolid and Acyclovir from Sequoia Research Products (Pangbourne, UK). Stock solutions were prepared by dissolving each compound in 50:50 v/v acetonitrile:water + 0.1% formic acid at concentrations ranging from 2500 – 10000 mg/L. Individual solutions for injection of each compound at 50 mg L^{-1} were prepared from stock, diluting with 95:5 v/v acetonitrile:100 mM ammonium formate pH 3.0. Toluene at 5 mg L^{-1} was used as a void volume marker and prepared in the same diluent. Water at 18.2 mΩ was supplied from a Millipore Milli-Q purifier (Watford, UK). Mobile phases were prepared gravimetrically based on the density of water and acetonitrile at room temperature.

2.2 Instrumentation and methodology

A Waters Acquity Classic Ultra Performance Liquid Chromatograph (UPLC, Waters Corp., Milford, USA) was used for all experiments, comprising of a binary solvent manager (BSM), sample manager (SM) and a diode array detector (DAD) equipped with a 500 nL flow cell. Data acquisition and hardware control was performed using Empower2 (Waters Corp., Milford, USA). The three superficially porous columns used (all Accucore HILIC) were bare silica, polymer coated amide and urea bonded, 2.6 µm particle size (shell thickness 0.5 µm, Thermo Scientific, Runcorn, UK) that were

kind gifts from the manufacturer. The column dimensions used throughout were 100 x 2.1 mm ID. Columns were operated using a flow rate of 0.4 mL/min and held at 30 ^{o}C for all experiments. 1.0 µL injections were made throughout using full loop injection mode. LogD values at $_{w}^{w}$pH 3 were calculated using the average of three different software packages: ACD/I-Lab (ACD Labs, Toronto, Canada), Marvin (ChemAxon, Budapest, Hungary) and MedChem Designer (Simulations-Plus, Lancaster, USA). Quoted pK_a values/values of solute charge were the average of results from the first two programs. Fig. 1 shows the structures and logD_{pH3} values for the compounds used in this study. Experiments on gradient retention time as a function of the re-equilibration time were performed according to the study of Carr *et al*. [28]. Briefly, this involved an initial sequence of six control gradients, five of which included 22.3 mins equilibration time, representing full equilibration (see 3.3). The sixth control run concluded with a specified equilibration time (e.g. 4.3 min). These runs were followed by four (n=4) experimental gradient runs at the same re-equilibration times. The experimental runs were followed by two control runs, the first of which used a full equilibration time (22.3 min.) and the second using the next equilibration time in the sequence (e.g. 7.3 min.) A further sequence of 4 experimental gradient runs was then performed, and the process repeated. Data were gathered for experimental re-equilibration times of 4.3, 7.3, 9.3, 12.3, 17.3, 22.3 and 27.3 minutes. The relative standard deviation (%RSD) was calculated for each compound in the gradient (tG=10 mins) after different re-equilibration times. Note that the equilibration time may well depend on the initial solvent composition as well as the range of concentration used during the gradient. The injection cycle time was 2.3 minutes, which was included in the stated re-equilibration times. This cycle time is quite long, being a consequence of the use of the full loop injection mode, which was employed to obtain maximum precision. Cycle times are typically much shorter for systems that used the flow through needle injection process [32]. The gradient time (tG) used throughout the study was always 10 minutes after which the mobile phase was immediately returned to the initial conditions. Mobile phases were typically flushed through the column for at least 1 hour prior to any experiments being performed. The mobile phases for gradient re-equilibration experiments were A: 95% ACN, 5 mM overall ammonium formate pH 3 and B: 60% ACN, 5 mM overall ammonium formate pH 3.

3. Results and discussion

3.1 Retention comparison between bare silica, amide and urea phases.

Isocratic retention data for the 20 structurally diverse antibiotic and antiretroviral compounds were collected at both 90% and 95% ACN containing 5 mM overall AF pH 3 on bare silica, amide and urea columns. 5 mM AF pH 3 was employed as this buffer concentration gives good peak shapes in HILIC [33,34] as opposed to sole use of formic acid (e.g. 0.1% v/v), despite the latter being favoured sometimes due to reduced suppression of solute signal intensity in electrospray mass spectrometry. Fig. 2 shows the large differences in *k* between the different columns. Clearly, HILIC is not a suitable procedure for linezolid, pyrazinamide, and chloramphenicol (which are neutral over the pH range 2-9) as their *k* < 1 on all columns. This result was not unexpected for linezolid and chloramphenicol, as their $logD_{pH3}$ values are > 0.5, indicating low hydrophilicity. Sulfamethoxazole was poorly retained under the conditions used, but has the possibility of using ionic interactions to increase retention, becoming negatively charged at pH >5. Pyrazinamide also has poor retention although is more hydrophilic ($logD_{pH3}$ -0.91) indicating that retention of this compound might be achieved solely by the partitioning mechanism. Isoniazid ($LogD_{pH3}$ -1.28), which is structurally similar to pyrazinamide gave appreciably higher retention on the three stationary phases. Its higher retention may be explained by its greater hydrophilicity and its positive charge in the mobile phase (estimated as +0.9), leading to the possibility of ionic interactions with ionised column silanols. In comparison, pyrazinamide was estimated to have zero charge in the mobile phase used. There is not always a good correlation between retention and logD values [12]. The correlation coefficients (*R*) of $logD_{pH3}$ versus log *k* (at 90% ACN) for all compounds on bare silica, amide and urea phases were only moderate at 0.67, 0.78 and 0.60 respectively, which further emphasises the difficulty in predicting retention in HILIC when considering a partition mechanism only. Moreover, there was disagreement between the predicted $logD_{pH3}$ values for pyrazinamide with -0.68, -1.23 and -0.80 being obtained from ACD, Marvin and MedChem Designer programs respectively. As noted previously [13], variation between predictive software packages further complicates retention correlation when using calculated log D values.

The highest retention factors observed on all phases were for meropenem and amoxicillin. These compounds are very hydrophilic with $logD_{pH3}$ values of -3.21 and -2.81 respectively. At a lower concentration of 90% ACN, meropenem was still very strongly retained on the bare silica phase (*k* = 63.1) although with much lower retention on the amide (*k* = 37.2) and significantly less on the urea phases (*k* = 10.9). Conversely, amoxicillin showed the strongest retention on the amide phase (*k* = 28.9) under the same conditions compared with the bare silica and urea phases. The data suggests that stronger ionic retention is experienced by meropenem (as ionic retention is high on bare silica phases). Indeed, the calculation programs suggest that meropenem may carry a slightly greater positive charge (+0.6) than amoxicillin (+0.5) at $_{w}^{w}$ pH 3.0, which in combination with its more

199 negative log D value, may explain its greater retention on silica. Amoxicillin and meropenem gave
200 excessive retention at 95% ACN on all of the columns, so *k* data was not obtained. The retention of
201 ganciclovir and acyclovir (neutral, nucleoside analogues) was stronger on the amide column than
202 with the bare silica column. It has been shown [9] that amide phases have significantly thicker water
203 layers than bare silica columns encouraging a partition retention mechanism. Interestingly, however,
204 retention for ganciclovir and acyclovir was only marginally larger on the urea phase than on bare
205 silica.

206 Fig. 3 indicates the correlation in *k* between the bare silica, amide and urea phases using 90%
207 ACN-buffer. Interestingly, the difference in selectivity of the bare silica versus the amide phase in
208 this study was smaller than data from previous findings [13], although this manufacturer's phases
209 have not been examined previously. The difference in selectivity when comparing amide and urea
210 phases was also small. Note however that the *R* values shown in the Figure should be treated with
211 some caution, as the points corresponding to higher *k* values are given much greater weight than the
212 other data points. However, larger differences in selectivity were found between the bare silica and
213 urea phases that can be explained by the very strong retention of meropenem, ciprofloxacin and
214 moxifloxacin on the former. Rifamycin compounds were reasonably well retained on both bare silica
215 and urea phases, whereas rifabutin had a $k < 1$ on the amide column. The selectivity factors (α) for
216 rifampicin and rifabutin on the urea and bare silica phases were 1.63 and 0.52 respectively. All of the
217 β-lactam and rifamycin antibiotics, except for amoxicillin and meropenem, were more retained on
218 the urea than on the amide phase. This might indicate some preferential selectivity from the urea
219 bonding towards structural features on these compounds. Surprisingly, flucytosine (neutral,
220 pyrimidine analogue) was retained more strongly on the urea phase than on either bare silica or the
221 amide phases. This might be considered unusual since the neutral guanosine-analogues acyclovir
222 and ganciclovir showed the strongest retention on the amide column, as seen previously with other
223 nucleosides in HILIC [13,33].

224 In summary, HILIC has been shown to be broadly applicable for the retention of antibiotic
225 and antiretroviral compounds using either bare silica, amide or urea bonded phases. Overall, using
226 95% ACN-buffer the bare silica column was the most retentive phase with an average *k* of the
227 antibiotic compounds of 9.6 compared with the amide and urea phases, which gave average *k* 6.4
228 and 4.3 respectively. However, due to the wide differences in retention it would be necessary to use
229 gradient elution to elute all retained compounds within a practical analysis time (see below).

230

3.2 Asymmetric peaks: addition of citrate

Poor peak shapes were seen on all phases for the fluoroquinolones ciprofloxacin and moxifloxacin as well as for d-cycloserine as illustrated for the bare silica phase in Fig. 4 when using 5 mM AF pH 3 in 90% ACN AF. Fig. 5 shows the same after the co-addition of citrate. All compounds showed some improvement in peak shape. For d-cycloserine, the tailing peak becomes almost symmetrical on this addition. Interestingly, there was a considerable reduction in retention (Fig. 6) for the fluoroquinolones upon co-addition of citrate, whereas very little change was seen for d-cycloserine. This reduction in retention may be due to citrate shielding strong secondary interactions complicit in the poor peak asymmetry seen with these compounds. Citrate is known to strongly chelate metal oxides in aqueous systems, particularly those of iron(III) [35]. While ethylene diamine tetraacetic acid (EDTA) might under some circumstances be a better complexing agent, it is rather insoluble in HILIC mobile phases containing high proportions of ACN [36]. We postulate that both fluoroquinolones and d-cycloserine undergo chelation-based interactions with labile metal oxides within the chromatographic apparatus. The quinolone group has been shown to have metal oxide chelation properties [37]. There are many potential sources of metal contamination within a chromatographic system. As shown by Euerby *et al*. [38,39] storage of columns in acetonitrile can result in the leaching of metals. Moreover, the presence of stainless steel column packing frits could also be significant, as their surface area is significantly larger in comparison to the wetted parts of metal connection tubing. Carr *et al*. [40] showed that corrosion of stainless steel column frits in acidic mobile phases results in the release of metal-oxides such as iron(II)/iron(III). Also, it has been shown elsewhere [41] that replacement of stainless steel frits with polyethylene-type were beneficial for the RP chromatography of metal-chelating phosphorylated compounds. It is therefore likely that available metal oxides become immobilised on silanol groups and thus act as metal affinity exchange sites. Several manufacturers now offer biocompatible instruments that are supposedly inert to metal oxide-solute interactions [42,43] which could be more suitable for the analysis of sensitive antibiotics. However, no previous reports on the interaction of d-cycloserine with metals could be found. A different explanation for the poor peak shape of d-cycloserine could be an on-column dimerization reaction, which becomes inhibited by the co-addition of citrate. It has been shown [44] that acetonitrile promotes the dimerization of d-cycloserine whereas this reaction is strongly inhibited in methanol. It is thought that methanol protects against nucleophilic attack on the carbonyl group through electrophilic solvation of the α-amino position.

It is possible that peak shapes might have been improved merely by increasing the concentration of ammonium formate buffer, although previous work did not demonstrate a strong dependence of peak shape on this parameter [45].

To summarise, fluoroquinolones showed evidence of stronger affinity towards system metal oxides in comparison to d-cycloserine that were not completely removed even after the co-addition of citrate. Therefore, the stainless steel column/frits/HPLC system used here might not be optimum for the analysis of fluoroquinolone antibiotics. Certainly, further work needs to be done to explore alternative operating conditions to further improve the peak shapes seen here for fluoroquinolones. An improved HILIC method for the analysis of these compounds could be useful as the $\log D_{pH3}$ values of ciprofloxacin and moxifloxacin are -2.19 and -1.46 respectively, indicating they are considerably hydrophilic.

3.3 Investigation of gradient re-equilibration in HILIC

As the *k* values of retained antibiotic and antiretroviral compounds were impractically different under isocratic conditions, we studied the use of gradient analysis. We chose bare silica and amide phases in this study with initial starting conditions of 95% ACN-buffer. The effect of buffer at both low (2 mM AF) and moderate (5 mM AF) concentrations was also investigated using the bare silica phase only. Equivalent buffer concentrations were maintained in both the A and B bottles to avoid introducing a salt concentration gradient. The test sample contained isoniazid, rifabutin, trimethoprim, flucytosine, acyclovir, ganciclovir and amoxicillin. Table 1 shows a summary of the repeatability of retention as a function of the different re-equilibration times for each of the investigated columns and conditions used. Notably, very good repeatability was observed regardless of the re-equilibration time, as long as the equilibration period was strictly the same between replicates, even for an equilibration time of only 4.3 min. This result is broadly in agreement with the work of Shollenberger and Bell and our previous studies in HILIC [31,32]. Carr *et al*. [28] also observed the same degree of repeatability under reversed-phase conditions. Fig. 7 shows chromatograms obtained at different gradient re-equilibration times on the bare silica column using 5 mM AF. It appears that while the retention of some peaks (e.g. amoxicillin (peak 7) is reasonably independent of equilibration time, the retention of others (e.g. Rifabutin (peak 2) shows considerably greater dependence. Figs. 8 a-c show the differences between the control value and the experimental retention times for each re-equilibration time and each solute. A positive or negative value on the y-axis indicates insufficient equilibration of the column (compared with “full equilibration”) resulting in a loss or gain of retention compared with the control run. The results in Fig. 7 and Fig. 8 are somewhat surprising, as it might be expected that insufficient equilibration of

the column would result universally in reduced solute retention times, as residues of the strong solvent remain in the column. For compounds that are neutral under the analysis conditions (flucytosine, acyclovir, ganciclovir), there were indeed losses in retention, which became worse with increasingly shorter re-equilibration times. Surprisingly, the basic compounds (rifabutin, trimethoprim) showed an increase in retention at shorter equilibration times, similar to previous findings [31,32]. Less divergence from the control run was seen for all compounds with the amide column. The situation is likely to be more complex in HILIC than RP, as changing the mobile phase from 95% ACN to a more aqueous composition results also in variation of the thickness of the water layer held at the stationary phase surface [8], as well as increasing the solvent strength. Furthermore, over the course of the gradient, the variation in the water layer thickness could result in changes in the distribution of buffer components away from the stationary phase surface into the bulk mobile phase region. It has been shown [13,21,34] that decreasing buffer concentration in HILIC results in reduced retention for neutral compounds, which is thought to be due to decreased thickness of the water layer. Alternatively, increased retention for basic compounds occurs at lower buffer concentration due to reduced competition for ion-exchange interactions [44]. These explanations correlate well with our results above.

A further factor influencing the equilibration process might be the absolute retention of each solute on the column. Only small changes in gradient retention time were observed for amoxicillin, which was the last eluting peak in the chromatogram on both columns under all conditions. We speculate that strongly retained compounds remain mostly immobile on the front of the column until the last stages of the gradient (at higher aqueous concentration values), and are thus unaffected by the exact equilibration state of the column at the start of a fresh gradient, when only a weak eluent is present. Similarly, the first peak (isoniazid) may be readily mobile through the column in a range of solvent compositions around that of the starting conditions (95% ACN–buffer), resulting again in approximately constant retention with equilibration time.

The data also indicates that bare silica and amide phases require a similar time/amount of mobile phase volume to have passed through in order to achieve full equilibration (Fig, 8). The data points converge into an asymptote indicating that full column equilibration has been established for each of the conditions. This point (22.3 mins) for the bare silica column represents around 40.5 column volumes (8.9 mL). Longer column equilibration (27.3 mins) seems unnecessary using these particular conditions, but can be strongly dependent on the nature of both the column and the mobile phase [32]. Furthermore, using lower buffer concentrations (Fig. 8 b-c) neither increased nor reduced the re-equilibration time needed for full equilibration to be achieved. Fig. 9 a-c show plots

of control-experimental gradient retention time against gradient retention time for the 7 solutes at the 7 different equilibration times. Fig. 9a clearly indicates that the differences between control and experimental retention on the amide column (as exhibited by the smaller spread of the diagonal lines) for the majority of solutes were much less affected by re-equilibration time than on the silica column (Fig. 9b and 9c). Overall, the basic compounds (rifabutin and trimethoprim) showed the greatest differences from the control runs on all columns and buffer conditions. This is perhaps unsurprising as the retention of basic compounds is more sensitive to possible transient alterations in buffer component distribution caused by the gradient than for pseudo-neutral or neutral compounds [34].

Finally, it would be possible to increase mobile phase flow during the equilibration step to further reduce equilibration time, as shown previously [32]. Note however, there did not seem to be a direct proportionality between equilibration time and flow rate in this step.

4. Conclusions

HILIC is broadly suitable for the analysis of many antibiotic and antiretroviral compounds with widely different physiochemical properties. The retention properties of three different superficially porous particle packed columns (bare silica, amide and urea bonded phases) were evaluated for these solutes under typical isocratic HILIC conditions. The selectivity, particularly of bare silica and urea phases, was different, indicating a useful degree of orthogonality for method development. There was much less difference in selectivity between the bare silica and amide phases. Fluoroquinolones and d-cycloserine gave severe peak tailing on all columns, attributable to on column metal-oxide interactions. This was likely due to chelation between the quinolone group (fluoroquinolones) or by on column dimerization (d-cycloserine) promoted by metal-oxides residing on the stationary phase. The co-addition of citrate proved moderately effective in improving peak shapes for fluoroquinolones, whereas the peak of d-cycloserine was considerably improved.

The wide differences in retention factors seen under isocratic conditions prompted an investigation into the effect of gradient re-equilibration time in HILIC. In order to obtain full equilibration in HILIC around 40 column volumes were needed between each run. Indeed, the time taken to achieve full equilibration was significantly longer than that needed to perform the separation (tG = 10 minutes, re-equilibration e.g = 22.3 minutes for the silica column). The progress of full equilibration was found to be largely unaffected by buffer concentration. Full equilibration time appears to be about twice that required in reversed-phase chromatography and is an obvious practical disadvantage of HILIC, with some consequences for gradient elution. Nevertheless, it was

shown that short gradient re-equilibration times of only a few minutes could be used with excellent retention repeatability, thus offering a practical solution to the problem. Separation selectivity in this “partial” or “pseudo-equilibrium” environment was shown to be considerably affected by equilibration time, which should therefore be held strictly constant for consistent results to be obtained.

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6. Legend to Figures

Fig. 1 Structures and $logD_{pH3}$ of test compounds.

Fig.2 Retention factors (k) for test compounds on bare silica, amide and urea phases using either 90% (top) or 95% (bottom) ACN AF $_w^w$pH3. Meropenem and amoxicillin not determined at 95% ACN due to excessively high retention.

Fig. 3 *k* versus *k* plots for different columns using 5 mM AF $_w^w$pH 3.0 in 90% ACN for bare silica, amide and urea phases.

Fig. 4 Chromatograms for moxifloxacin (a) ciprofloxacin (b) and cycloserine (c) on the bare silica phase. Mobile phase: 90% ACN, 5 mM overall ammonium formate adjusted to $_w^w$pH 3 with formic acid. Moxifloxacin λmax = 295 nm, ciprofloxacin λmax = 280 nm and cycloserine λmax = 215 nm.

Fig. 5 Chromatograms for moxifloxacin (a) ciprofloxacin (b) and cycloserine (c) on the bare silica phase after the co-addition of citrate. Mobile phase: 90% ACN, 2.5 mM overall ammonium formate, 2.5 mM ammonium citrate adjusted to $_w^w$pH 3 with formic acid.

Fig. 6 Retention factor (a), asymmetry factor (b) and peak efficiency (c) measurements for moxifloxacin, ciprofloxacin and d-cycloserine on the bare silica column using 90% ACN with either AF or AF/AC.

Fig. 7 Chromatograms obtained of the gradient test mix on the bare silica column using different re-equilibration times. Linear gradient from 100% A to 70% A, 5 mM buffer (effectively 95% ACN-buffer to 84.5% ACN-buffer) in 10 min. Peak identities: (1) Isoniazid (2) Rifabutin (3) Trimethoprim (4) Flucytosine (5) Acyclovir (6) Ganciclovir (7) Amoxicillin. λmax = 275 nm.

Fig. 8 Effect of re-equilibration time on the difference between experimental and control gradient retention times. (a) amide Linear gradient from 100% A to 60% A, 5 mM buffer(effectively 95 % ACN-buffer to 81 % ACN-buffer) in 10 min. (b) bare silica Linear gradient from 100% A to 70% A, 5 mM buffer) (c) bare silica (Linear gradient from 100% A to 70% A, 2 mM buffer). The mobile phases used were as indicated in section 1.2.

Fig. 9 Effect of gradient re-equilibration time on the difference between experimental and control gradient retention times as a function of retention time. Conditions were as in Fig. 8 for (a), (b) and (c) respectively.

%RSD (n = 4) Amide (5 mM)

Re-Eq. Time (mins)	Rifabutin	Isoniazid	Trimethoprim	Flucytosine	Acyclovir	Ganciclovir	Amoxicillin
4.3	0.080	0.046	0.039	0.034	0.134	0.218	0.094
7.3	0.047	0.048	0.041	0.056	0.100	0.121	0.077
9.3	0.152	0.024	0.149	0.062	0.079	0.101	0.076
12.3	0.459	0.067	0.392	0.079	0.076	0.062	0.045
17.3	0.123	0.013	0.114	0.018	0.023	0.033	0.032
22.3	0.014	0.013	0.020	0.015	0.003	0.006	0.004
27.3	0.007	0.009	0.017	0.051	0.044	0.041	0.031

%RSD (n = 4) Bare silica (5 mM)

Re-Eq. Time (mins)	Rifabutin	Isoniazid	Trimethoprim	Flucytosine	Acyclovir	Ganciclovir	Amoxicillin
4.3	0.102	0.335	0.113	0.028	0.057	0.077	0.200
7.3	0.249	0.323	0.246	0.043	0.176	0.307	0.158
9.3	0.123	0.192	0.216	0.028	0.168	0.066	0.137
12.3	0.187	0.135	0.041	0.070	0.177	0.086	0.125
17.3	1.028	0.123	0.841	0.024	0.030	0.037	0.068
22.3	0.056	0.044	0.043	0.030	0.017	0.012	0.005
27.3	0.070	0.014	0.045	0.019	0.019	0.027	0.036

%RSD (n = 4) Bare silica (2 mM)

Re-Eq. Time (mins)	Rifabutin	Isoniazid	Trimethoprim	Flucytosine	Acyclovir	Ganciclovir	Amoxicillin
4.3	0.094	0.159	0.109	0.034	0.052	0.053	0.250
7.3	0.256	0.116	0.269	0.040	0.055	0.071	0.190
9.3	0.145	0.068	0.135	0.040	0.050	0.078	0.163
12.3	0.109	0.114	0.098	0.025	0.033	0.056	0.119
17.3	0.124	0.064	0.320	0.040	0.157	0.020	0.223
22.3	0.035	0.056	0.026	0.027	0.021	0.017	0.011
27.3	0.036	0.039	0.035	0.040	0.034	0.036	0.066

Table 1 Relative standard deviation (RSD) of gradient retention time using different re-equilibration times on amide and bare silica columns.

Fig. 3

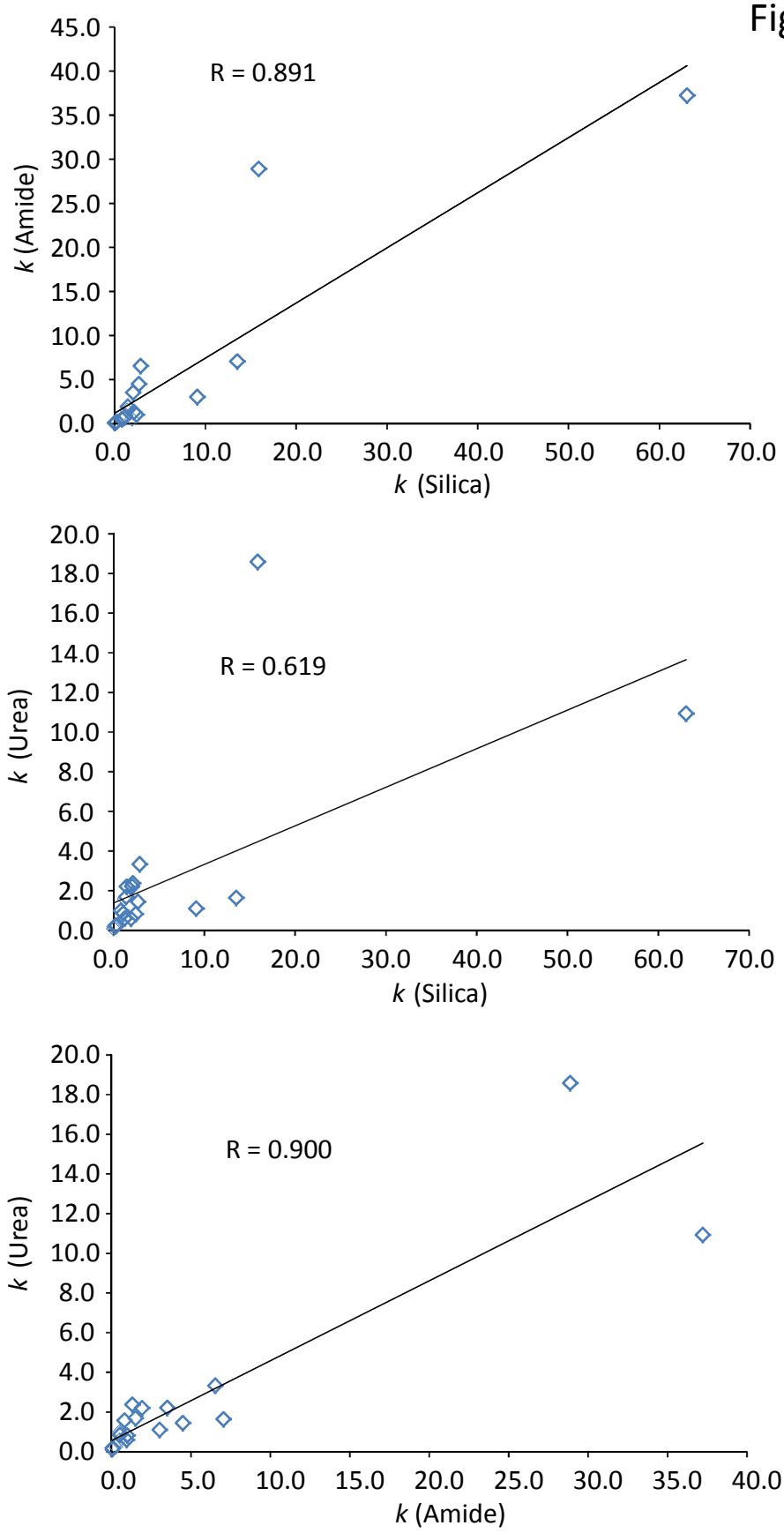
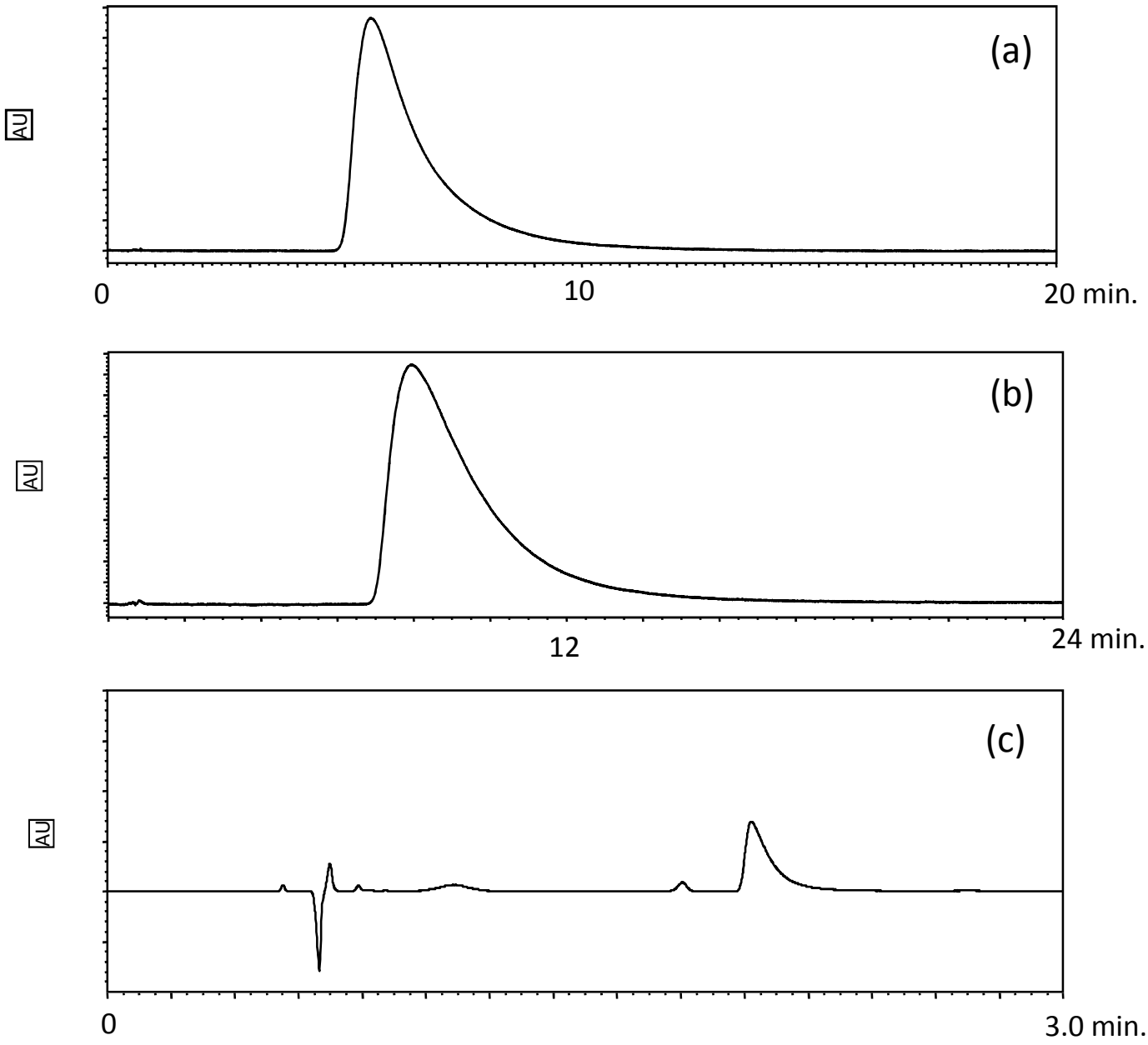
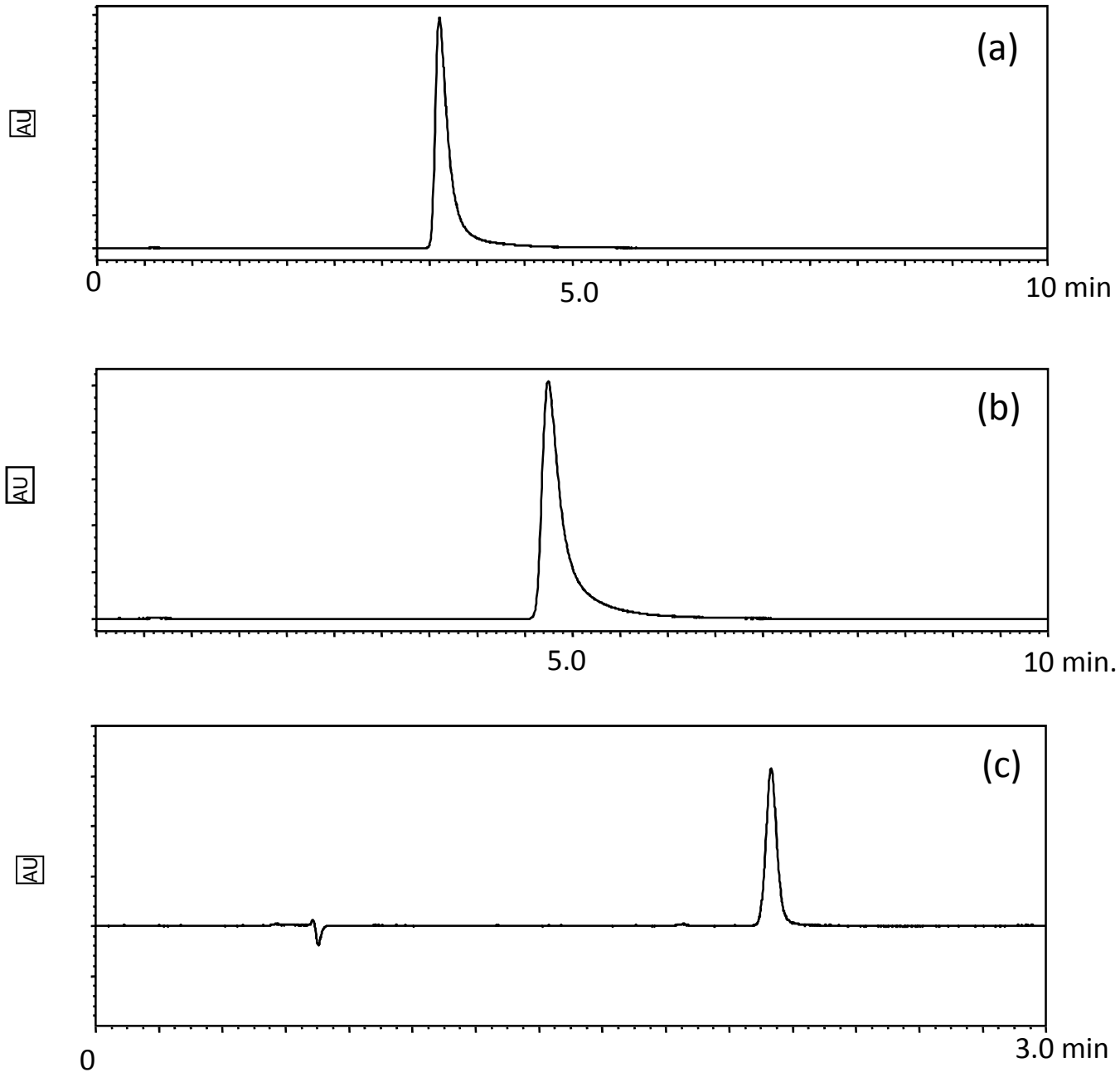

R = 0.891
k (Amide)
k (Silica)
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
R = 0.619
k (Urea)
k (Silica)
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
R = 0.900
k (Urea)
k (Amide)
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0

Fig. 4




(a)
AU
0
5.0
10 min
(b)
AU
5.0
10 min.
(c)
AU
0
3.0 min

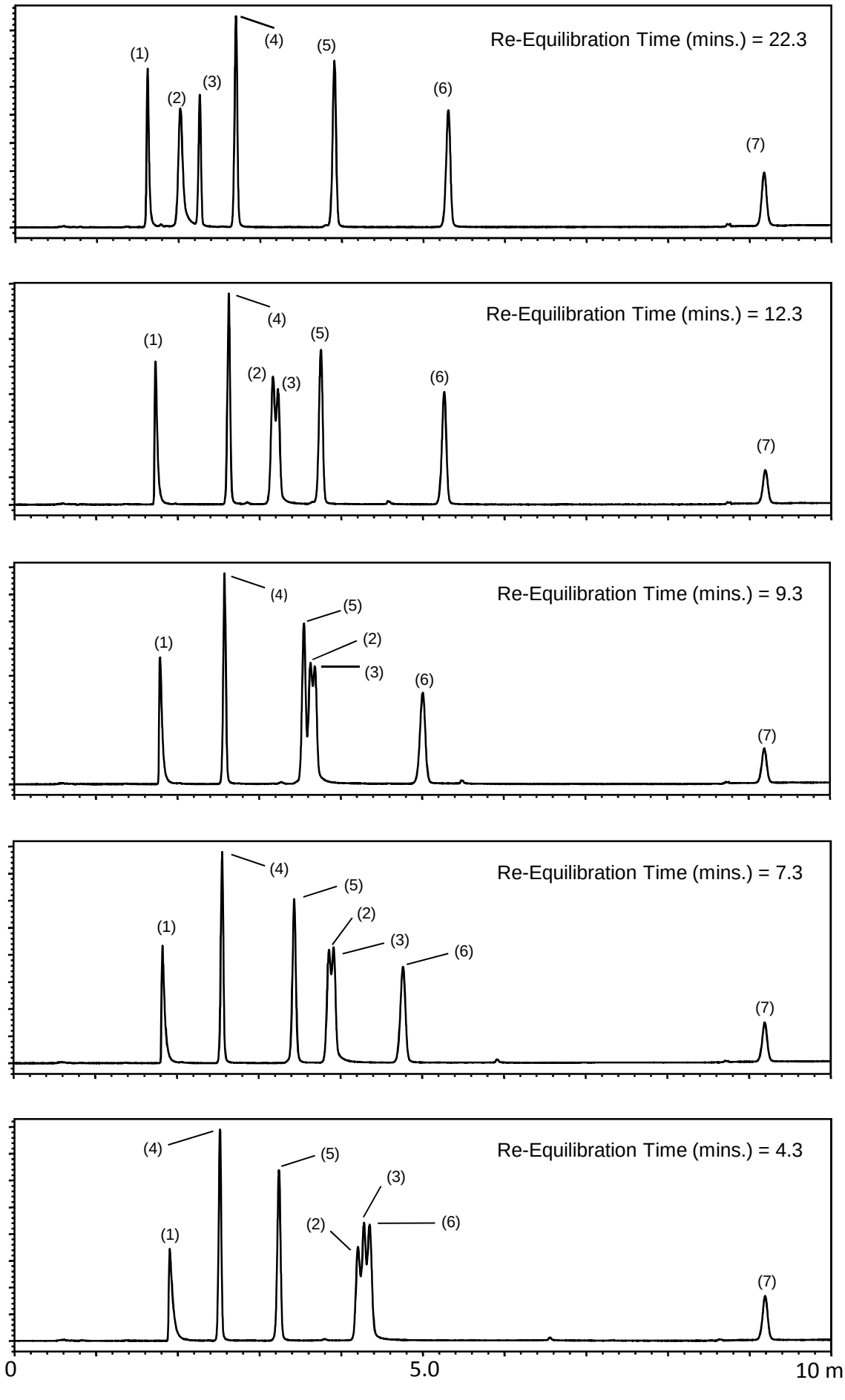

Re-Equilibration Time (mins.) = 22.3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
Re-Equilibration Time (mins.) = 12.3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
Re-Equilibration Time (mins.) = 9.3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
Re-Equilibration Time (mins.) = 7.3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
Re-Equilibration Time (mins.) = 4.3
(1)
(2)
(3)
(4)
(5)
(6)
(7)
0
5.0
10 min.

Fig. 7

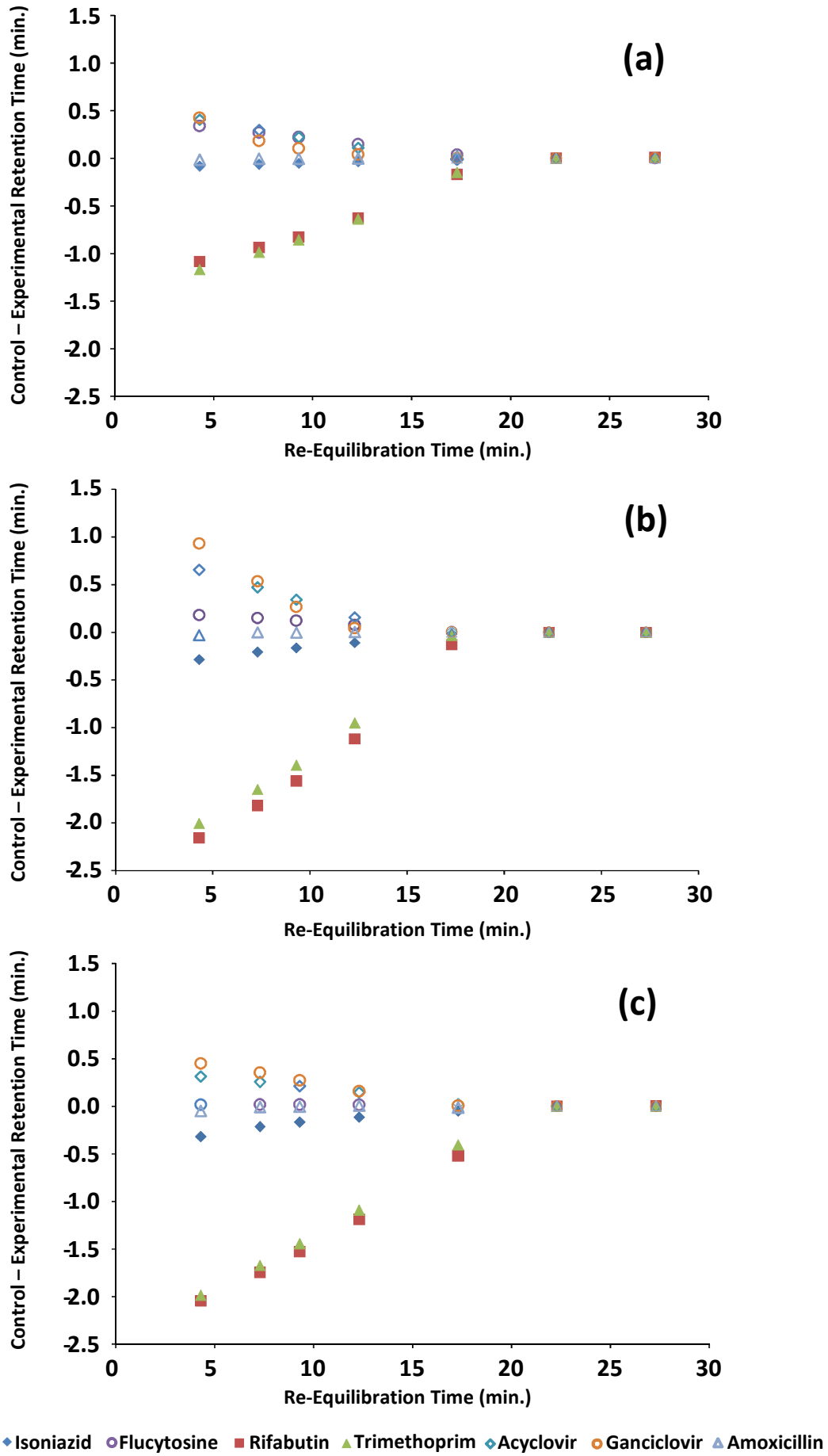
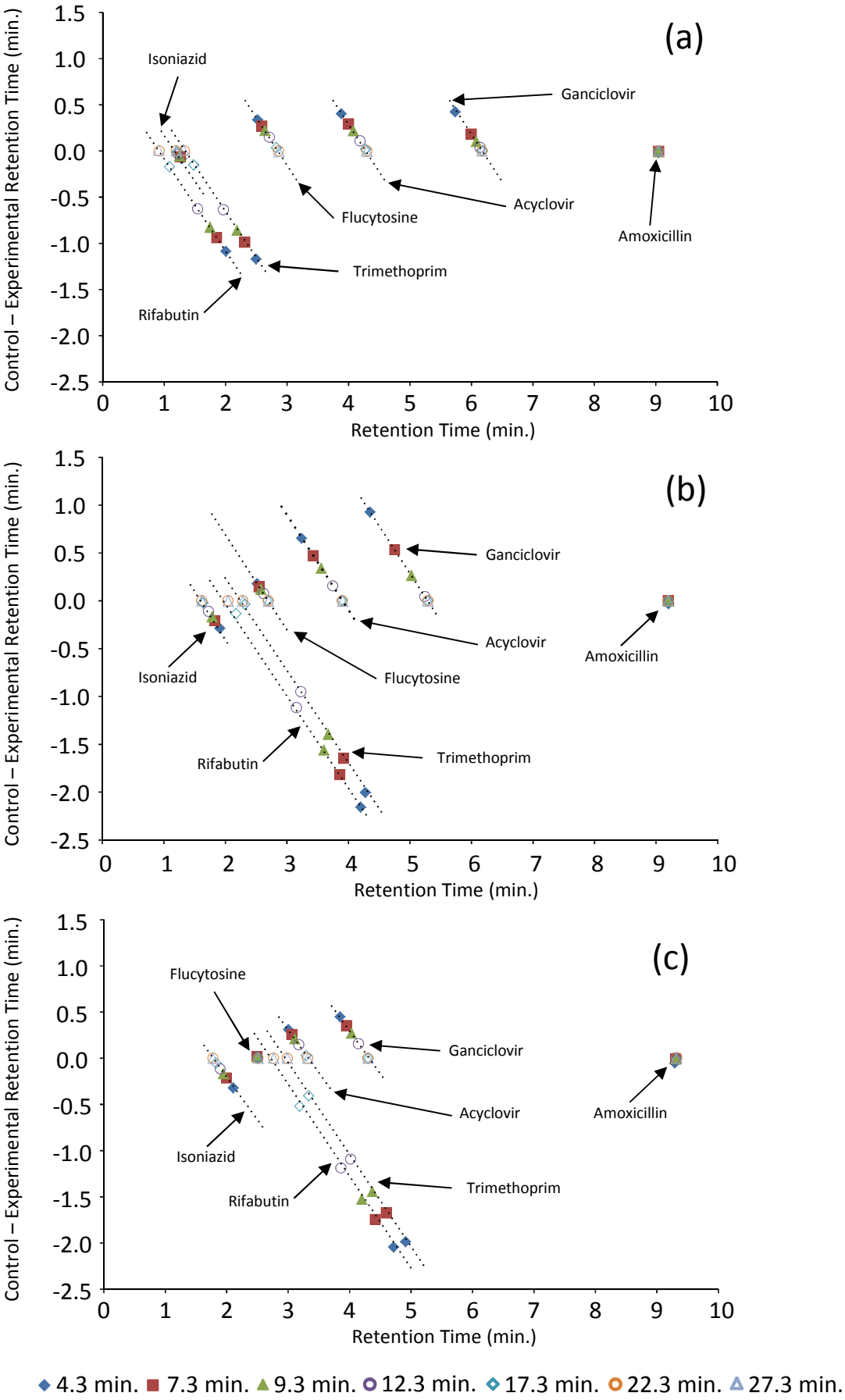
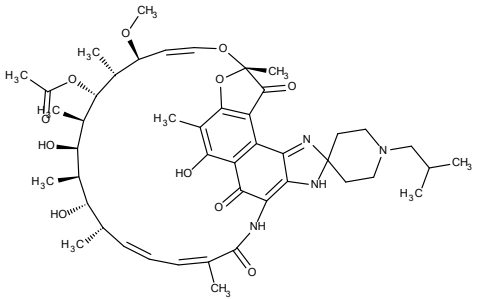
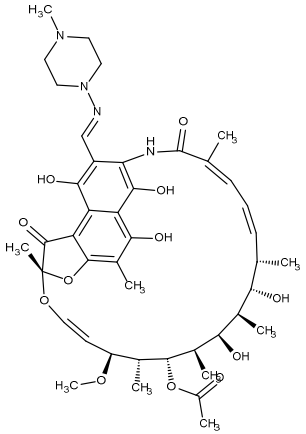

(a)
(b)
(c)
Control – Experimental Retention Time (min.)
Re-Equilibration Time (min.)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
5
10
15
20
25
30
Isoniazid
Flucytosine
Rifabutin
Trimethoprim
Acyclovir
Ganciclovir
Amoxicillin

Figure 8

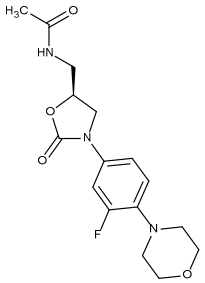

(a)
Isoniazid
Ganciclovir
Flucytosine
Acyclovir
Amoxicillin
Trimethoprim
Rifabutin
Control – Experimental Retention Time (min.)
Retention Time (min.)
(b)
Ganciclovir
Acyclovir
Amoxicillin
Isoniazid
Flucytosine
Rifabutin
Trimethoprim
Control – Experimental Retention Time (min.)
Retention Time (min.)
(c)
Flucytosine
Ganciclovir
Acyclovir
Amoxicillin
Isoniazid
Rifabutin
Trimethoprim
Control – Experimental Retention Time (min.)
Retention Time (min.)
4.3 min. 7.3 min. 9.3 min. 12.3 min. 17.3 min. 22.3 min. 27.3 min.



Rifabutin
$logD_{pH3}$ 0.58



Rifampicin
$logD_{pH3}$ 0.83



Linezolid
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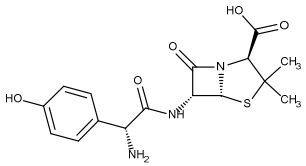

Amoxycillin
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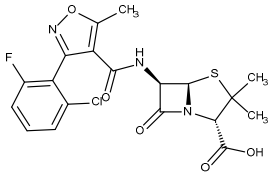
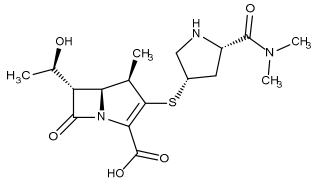
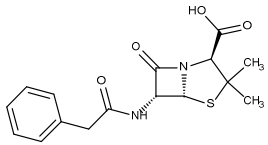


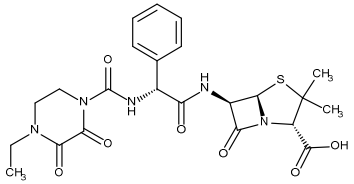
Flucloxacillin
$logD_{pH3}$ 2.41



Meropenem
$logD_{pH3}$ -3.21



Penicillin G
$logD_{pH3}$ 1.28



Piperacillin
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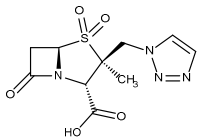
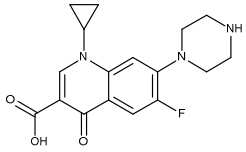


Tazobactam
$logD_{pH3}$ -2.02



Ciprofloxacin
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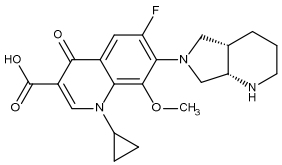
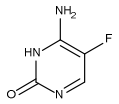
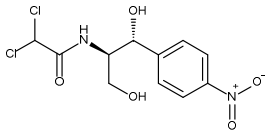


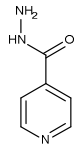
Moxifloxacin
$logD_{pH3}$ -1.46



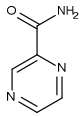
Flucytosine
$logD_{pH3}$ -1.08



Chloramphenicol
$logD_{pH3}$ 0.97

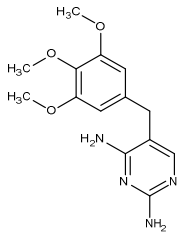


Isoniazid
$logD_{pH3}$ -1.28

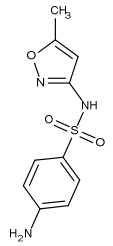


Pyrazinamide
$logD_{pH3}$ -0.91

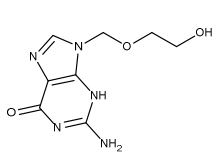
Cycloserine
$logD_{pH3}$ -3.43



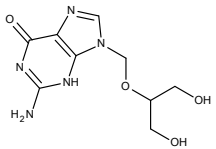
Trimethorprim
$logD_{pH3}$ -1.49



Sulfamethoxazole
$logD_{pH3}$ 0.77

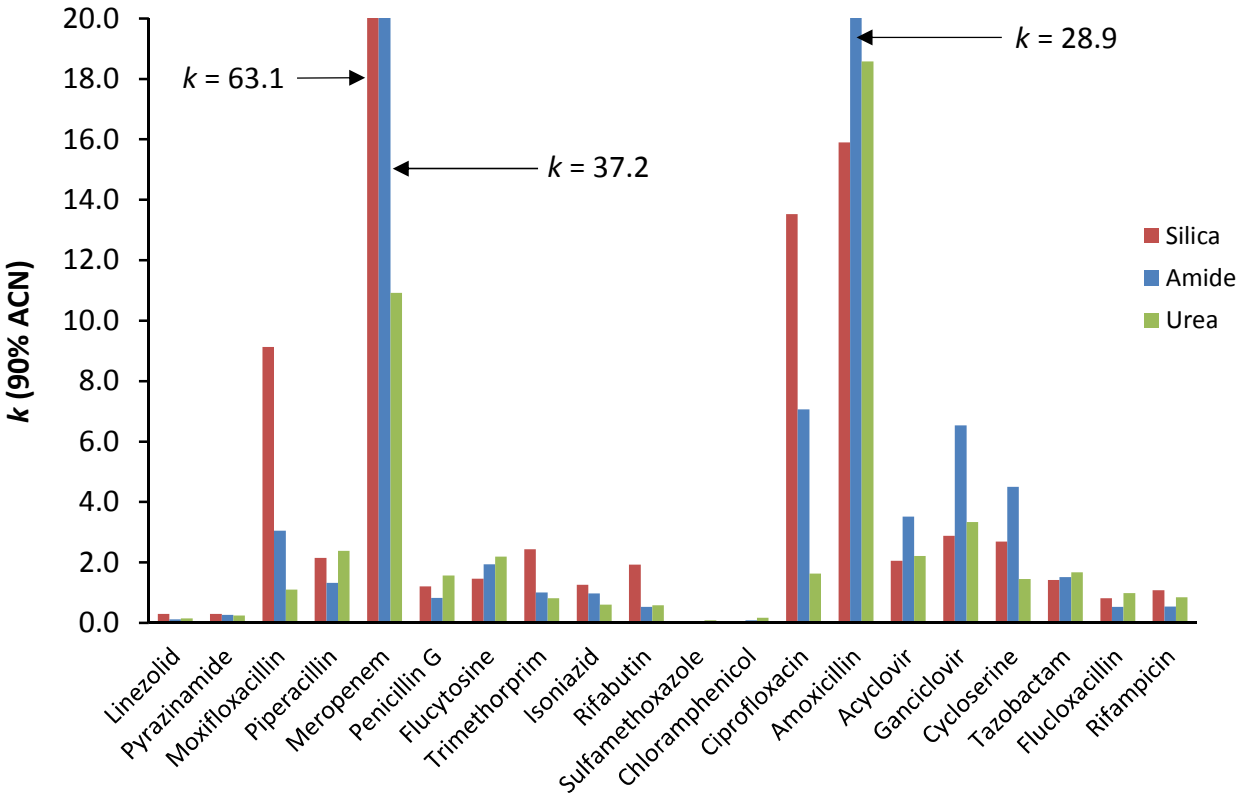


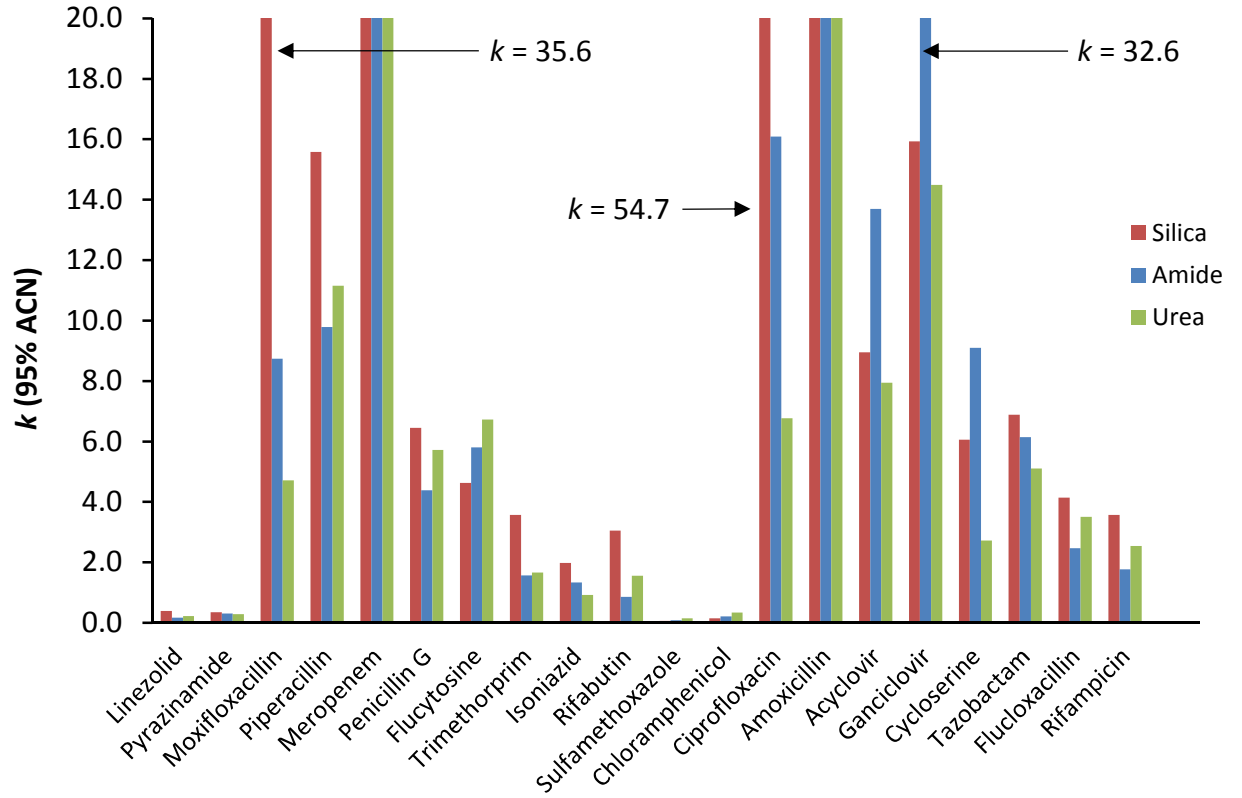
Aciclovir
$logD_{pH3}$ -1.39

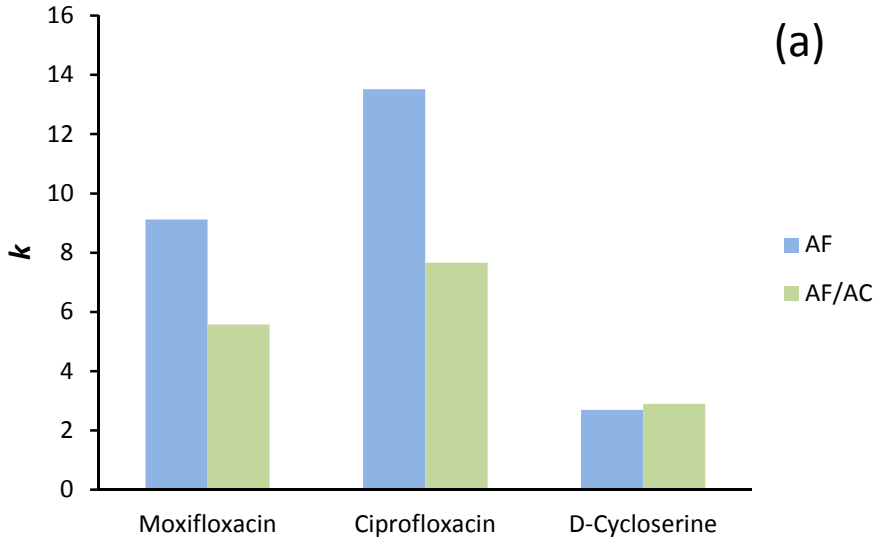


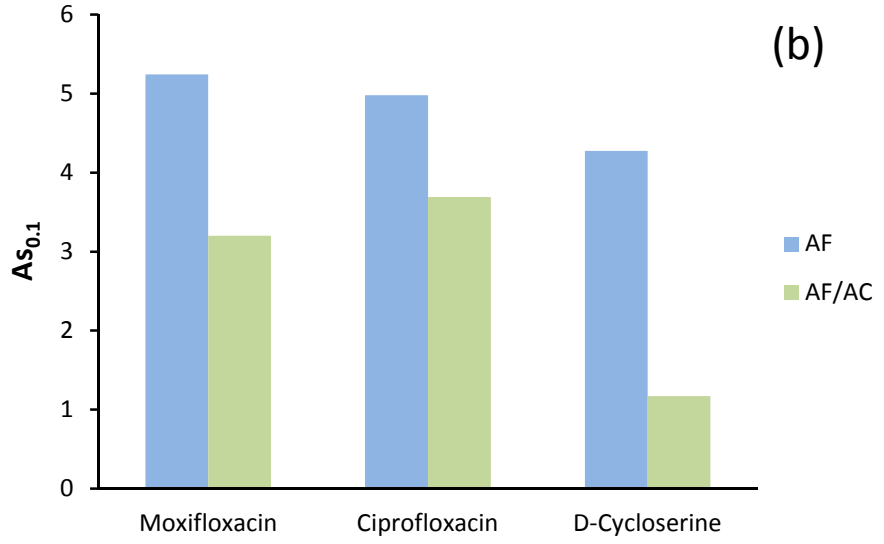
Ganciclovir
$logD_{pH3}$ -2.00

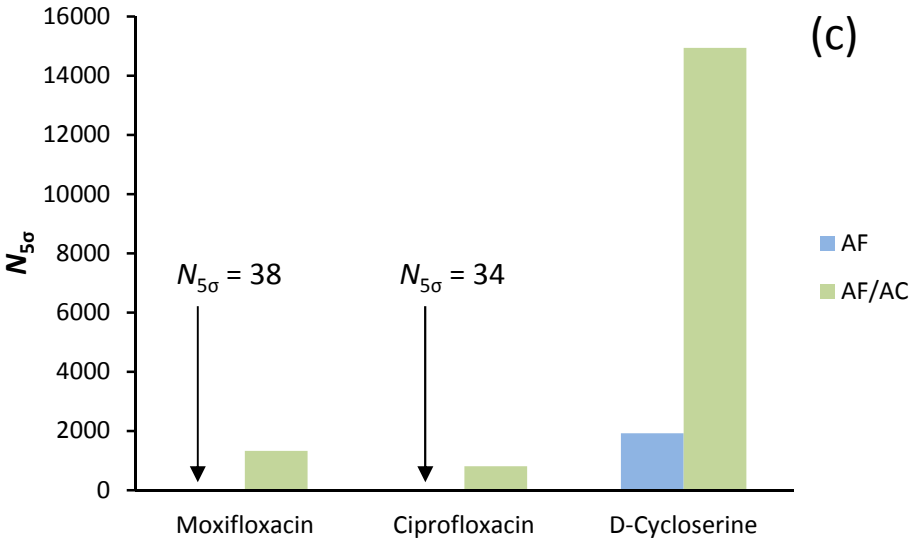
Figure 1


k (90% ACN)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
k = 63.1
k = 37.2
k = 28.9
Silica
Amide
Urea
Linezolid
Pyrazinamide
Moxifloxacillin
Piperacillin
Meropenem
Penicillin G
Flucytosine
Trimethorprim
Isoniazid
Rifabutin
Sulfamethoxazole
Chloramphenicol
Ciprofloxacin
Amoxicillin
Acyclovir
Ganciclovir
Cycloserine
Tazobactam
Flucloxacillin
Rifampicin


k (95% ACN)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
k = 35.6
k = 54.7
k = 32.6
Silica
Amide
Urea
Linezolid
Pyrazinamide
Moxifloxacillin
Piperacillin
Meropenem
Penicillin G
Flucytosine
Trimethorprim
Isoniazid
Rifabutin
Sulfamethoxazole
Chloramphenicol
Ciprofloxacin
Amoxicillin
Acyclovir
Ganciclovir
Cycloserine
Tazobactam
Flucloxacillin
Rifampicin


(a)
16
14
12
10
8
6
4
2
0
k
AF
AF/AC
Moxifloxacin
Ciprofloxacin
D-Cycloserine


(b)
6
5
4
3
2
1
0
As0.1
AF
AF/AC
Moxifloxacin
Ciprofloxacin
D-Cycloserine


(c)
16000
14000
12000
10000
8000
6000
4000
2000
0
N5σ
N5σ = 38
N5σ = 34
AF
AF/AC
Moxifloxacin
Ciprofloxacin
D-Cycloserine

- HILIC is a suitable method for HPLC analysis of a wide range of antibiotics.
- Selectivity of analysis can change dependent on the stationary phase.
- Citrate improves peak shape of some solutes by reducing metal oxide interactions.
- Full equilibration times are much longer in HILIC than in RP.
- Repeatable partial equilibration in gradient elution achieved in < 5 min.